



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 152 746
A2

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 85100224.6

(22) Date of filing: 11.01.85

(51) Int. Cl.⁴: **C 12 N 5/00, C 12 P 21/00,**
C 12 N 15/00
// C07K3/18,
G01N33/577, (C12P21/00,
C12R1:91)

(30) Priority: 26.10.84 US 664919
12.01.84 US 570082
07.01.85 US 689274

(43) Date of publication of application: 28.08.85
Bulletin 85/35

(54) Designated Contracting States: AT BE CH DE FR GB IT
LI LU NL SE

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(54) Monoclonal antibodies to factor VIIIc.

(57) Hybridomas producing monoclonal antibodies specific for polypeptide fragments derived from human Factor VIIIc are provided. Class I hybridomas produce monoclonal antibodies reactive with a 80/77 kd doublet fragment or with both the 80/77 kd doublet and a 240 kd polypeptide. Class III hybridomas produce monoclonal antibodies reactive with the 240 kd polypeptide as well as a 92.5 kd fragment and its precursors. Class III antibodies show additional reactivity with a 40 kd thrombin digestion product. The monoclonal antibodies are useful for the separation of Factor VIIIc and its constituent polypeptides, as well as for the immunoassay of Factor VIIIc in biological samples.

Cell lines 42, 47, 56 and I-28 were deposited at National Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts, U.K. on December 21, 1984, and granted accession nos. 84122103, 84122102, 84122104 and 84122101 respectively.

Cell lines 4B5, 5B1, and 2B6 were deposited at the American Type Culture Collection on December 27, 1984, and granted accession nos. HB 8688, HB 8689 and HB 8690 respectively.

ACTORUM AG

9729-20-2

MONOCLONAL ANTIBODIES TO FACTOR VIIIC

This application is a continuation-in-part of application serial no. 664,919, filed October 26, 1984, which is a continuation-in-part of application serial no. 570,062 filed January 12, 1984.

BACKGROUND OF THE INVENTIONField of the Invention

Factor VIIIC is a plasma protein that participates in the intrinsic pathway of blood coagulation. It is absent or defective in individuals with the hereditary X chromosome-linked recessive bleeding disorder hemophilia A. Great difficulty has been encountered in isolating Factor VIIIC due to its extremely low concentration in plasma and the fact that it appears to be an intermediate or final degradation product of a larger protein precursor. Efforts to isolate Factor VIIIC have led to complex mixtures of various polypeptides having significant heterogeneity and varying molecular weights.

It would be of great interest therefore to prepare monoclonal antibodies which are capable of selectively reacting with individual polypeptides or groups of polypeptides which are derived from Factor VIIIC.

Description of the Prior Art

U.S. Patent No. 4,361,509 and references cited therein describe purification of Factor VIIIC. See also Fulcher and Zimmerman, Proc. Natl. Acad. Sci. USA (1982) 79:1648-1652. Tuddenham et al., J. of Lab. Clinical Medicine (1979) 93:40-53 describes purification of Factor VIIIC using polyclonal antibodies. Austen, British J. Hematology (1979) 43:669-674 describes the use of aminohexyl-Sepharose for Factor VIIIC purification. Weinstein et al., Proc. Natl. Acad. Sci. USA (1981) 78:5137-5141 describes a study of the effect of thrombin on Factor VIIIC. See also Kuo

et al., Abstracts for IX International Congress of Thrombosis and Hemostasis, (Stockholm; July, 1983).

Hybritech Data Sheet (Hybritech, Inc., 11085 Torreyana Road, San Diego, California 92121) describes
5 a monoclonal antibody (Catalog #0432) which is specific for human Factor VIIIC. Danish patent application 53519 corresponding to U.S. patent applications 481,103 and 556,508 describes the preparation of monoclonal
10 antibodies to human factor VIIIC and polypeptide fragments thereof. Certain of the monoclonal antibodies described in the Danish patent application are also described by Zimmerman et al. (1983) Blood 61:807. Fass et al. (1982) Blood 59:594, report that active
15 human Factor VIIIC may be eluted from monoclonal antibodies by ethylene glycol (50%).

SUMMARY OF THE INVENTION

Particular hybridoma cell lines producing monoclonal antibodies reactive with human Factor VIIIC are provided. Monoclonal antibodies produced by cell
20 lines 42, 47 and 56 are reactive with a 80/77 kd doublet obtained by electrophoresis of Factor VIIIC material under denaturing conditions. Monoclonal antibodies produced by cell lines 1B9, 2B6 and 2F6 react with the 80/77 kd doublet and with a 240 kd
25 fragment. Monoclonal antibodies produced by cell lines 4E5 and 5B1 react with the 240 kd fragment, the 92.5 kd fragment and its precursors, as well as a 40 kd thrombin digestion product of Factor VIIIC material. Of the above, only antibodies produced by 56 cell lines
30 displayed coagulation inhibition.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Hybridomas producing monoclonal antibodies reactive with different polypeptide fragments of human Factor VIIIC are provided. Class I hybridomas produce
35 monoclonal antibodies reactive with a 80/77 kd doublet fragment of Factor VIIIC or with both a 240 kd fragment and a 80/77 kd doublet. Class III hybridomas produce

monoclonal antibodies reactive with the same 240 kd fragment as well as a 92.5 kd fragment and precursors thereof. Class III antibodies show an additional reactivity with a 40 kd thrombin digestion product of Factor VIIIC material.

Human Factor VIIIC is a complex protein which can be isolated in substantially pure form and exhibits an apparent molecular weight of about 460kd. Upon electrophoresis under denaturing conditions, a large number of fragments of varying molecular weights result: 240, 160, 140, 115, 92.5, 80 and 77kd, the latter two being found to migrate as a doublet. Analysis of the fragments by chemical and protease cleavage including thrombin, employing the monoclonal antibodies of the present invention to follow immunogenic relationships and cleavage patterns to follow structural relationships, demonstrates that the 92.5kd polypeptide is related to the 240, 160, 140 and 115 polypeptides while the 77/80 doublet appears to have common antigenic characteristics with only the 240kd polypeptide. It is further found that the 77/80kd doublet is converted by thrombin to a 67/70kd doublet, while the 92.5kd polypeptide present in purified Factor VIIIC material treated with thrombin is cleaved by thrombin, directly or indirectly, into two polypeptides of about 40 and 52.5kd.

The monoclonal antibodies of the present invention are useful for a variety of purposes. For example, the antibodies may be used to isolate the entire Factor VIIIC complex from plasma, as well as isolating the various polypeptide fragments of Factor VIIIC from a purified source. In addition, the antibodies may be used for detecting the presence of Factor VIIIC and related polypeptides in plasma by a variety of immunoassay techniques. Suitable immunoassay techniques are well known and amply described in the scientific and patent literature.

The monoclonal antibodies of the present invention may also be used to facilitate preparation of additional hybridomas producing monoclonal antibodies having substantially identical reactivity, but often
5 differing in other characteristics, such as isotype, binding affinity, and the like. This can be achieved by using the monoclonal antibody to enrich these polypeptides carrying determinant sites recognized by the antibody in a source Factor VIIIC. Conveniently,
10 an affinity column may be prepared using the desired antibody. By then applying the Factor VIIIC to the column in an appropriate buffer, polypeptides recognized by the antibody will be bound. After washing the column to assure removal of
15 non-specifically bound proteins, the polypeptide of interest may be released from the column by adjusting the pH, employing urea, or by utilizing other mildly denaturing conditions. The antigen may then be isolated and used to hyperimmunize an appropriate host,
20 and hybridomas produced by the now classical teachings of Kohler and Milstein (1975) Nature 256:495, or more recent adaptations thereof. Details of suitable immunization, immortalization, and screening procedures are set forth below. Following such procedures, a
25 variety of antibodies having substantially identical reactivities, but differing in class, binding constant, host, and the like, can be obtained.

In general, hybridomas may be produced by first hyperimmunizing a mouse or other small mammal
30 with the Factor VIII polypeptide of interest. Specific methods of immunization are well known and are amply described in the literature. The Factor VIII polypeptide is injected with or without adjuvants into the mammal followed by from 2 to 6 subsequent booster
35 injections over a relatively short period of time. The animals are then killed, usually several days after the

final injection, the spleens removed, and the spleen cells immortalized.

The manner of immortalization is not critical. Presently, the most common way is fusion with a myeloma cell fusion partner, which method is exemplified here. Other techniques include EBV transformation, transformation with bare DNA, e.g., oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies.

When employing fusion with fusion partner, the manner of fusion is not critical and various techniques may be employed. Conveniently, the spleen cells and myeloma cells are combined in the presence of polyethylene glycol, a suitable growth medium, and other additives for a few minutes. At the end of the fusion, the non-ionic detergent is removed by washing the cells.

The fused cells are then promptly dispensed into small culture wells, usually in a microtiter plate, at relatively low density for screening. The growth medium in the culture wells is selected to support growth of the hybrid cells while being lethal to the myeloma cells. Conveniently, the myeloma cell line has been mutated to be sensitive to particular agents, typically being HAT sensitive.

After sufficient time, usually from one to two weeks, colonies of hybrids are observed and plates containing positive wells are identified. The plates and wells having only one colony per well are selected, and the supernatants from these wells are tested for binding activity against the particular polypeptide fragment(s) of Factor VIIIC. Other characteristics of the hybridoma cell line and monoclonal antibodies, such as growth characteristics of the hybridoma, binding affinity of the monoclonal antibodies, antibody class, cross-reactivity of the antibodies, and the like, can

then be determined. Those hybridomas which produce antibodies having the desired specificity as well as other desirable characteristics can then be maintained as viable cultures and/or frozen storage.

5 The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL

1. Purification of Factor VIIIC

10 Human Factor VIIIC was isolated from commercial cryoprecipitate preparations by a) immunosorbent chromatography using a polyclonal anti VIIIR-Sepharose column by a method first described by E.G.D. Tuddenham, N.C. Trabold, J.A. Collins, and L.W. Hoyer, J. of Lab. Clinical Medicine (1979) 93:40; and b) a chromatographic separation on aminohexyl-substituted agarose as
15 was originally described by D.E.G. Austen, British J. of Hematology (1979) 43:669. Details of the procedures are described below.

20 Goat anti-human Factor VIII Related Antigen (VIIIR) serum obtained from Atlantic Antibody (cat. no. 040-01), was treated with either a standard 0-50% ammonium sulfate cut followed by DEAE cellulose column chromatography, or a similar 0-33% cut without subsequent chromatography. These materials were then
25 conjugated to CNBr-activated Sepharose CL2B or 4B, respectively, (Pharmacia, 17-0140-01 or 17-0430-01) and poured as a column (anti VIIIR-Sepharose column).

30 "HEMOFIL", a stable, dried preparation of antihemophilic factor (Factor VIII, AHF, AHG) in concentrated form prepared from fresh, normal human plasma, representing about a 40-fold enrichment for Factor VIIIC, was dissolved in the following buffer: 0.02M imidazole, 0.15M NaCl, 0.1M lysine-HCl, 0.02% NaN₃, pH 7.4.

35 After being dissolved, the Hemofil was applied to the above-described anti VIIIR-Sepharose column. Non-specifically bound protein was eluted with

the above buffer modified to 0.5M NaCl. Next, Factor VIIIC was eluted with the above buffer containing 0.35M CaCl_2 , with the addition of 10% glycerol which stabilizes the Factor VIIIC activity. Active fractions from the immunosorbent column were pooled and dialyzed against buffer (0.02M imidazole, 0.15M NaCl, 0.1M lysine-HCl, 0.025M CaCl_2 , 0.02% NaN_3 , 10% glycerol, pH 7.4). An aliquot of the dialyzed fractions, which contained 1,100 units of Factor VIIIC, was applied to an aminohexyl-Sepharose 4B column (1x6cm) equilibrated with dialysis buffer described above. Factor VIIIC activity was eluted with the same buffer containing either 0.35M CaCl_2 or 2M NaCl. The activity was found to be in a volume of 2ml with 500 units of Factor VIIIC per ml. Subsequent experiments carried out in the same manner provided a recovery of 25% off the anti VIIIR column and a recovery of approximately 90% off the aminohexyl column. Alternatively, pooled, dialysed material eluted from the immunosorbent column is first applied to a dextran sulfate agarose (Pharmacia) column (1.5 x 6cm) equilibrated with the dialysis buffer above and eluted with the same buffer. Several minor contaminants, e.g., fibrinogen, fibronectin, IgG, are retained on the column while Factor VIIIC emerges in the flow-through which is collected and loaded on the aminohexyl-Sepharose column as before.

Both biological, i.e., clotting, and antigenic (CAG) activity were shown to be present in the purified Factor VIIIC, as demonstrated by the subsequent assays indicating a 5,000-fold purification over the 40-fold concentration in Hemofil. Using a standard commercially available three component kit from General Diagnostics, (APTT, Factor VIII deficient plasma, Verify Normal Citrate) a coagulation assay was carried out and indicated high levels of Factor VIIIC biological activity.

2. Preparation of Monoclonal Antibodies

Balb/c mice were immunized with purified human Factor VIIIC, as described above. Spleen cells (10^8) were fused with 10^7 NSO or 10^7 NSI mouse myeloma cells (according to Kohler and Milstein (1975) Nature 256:495). The products of each fusion were plated onto two 96 well microtiter plates. A spleen cell feeder layer was used at 10^4 cells/well. Colonies were visible microscopically from the fifth day, and the supernatants were assayed every few days for presence of antibodies against Factor VIIIC. An enzyme linked immunoadsorbent assay (ELISA) was used employing the following layers in an immunoassay plate (Nunc F2):

- 1st layer: Monospecific anti-mouse Ig
- 2nd layer: Hybridoma cell supernatant
- 3rd layer: Cryo-precipitate containing human Factor VIIIC
- 4th layer: Peroxidase labelled IgG from a hemophilic inhibitor patient.

Four positive cell lines were identified, purified by subcloning and grown in liter scale cultures in the laboratory. Three of the positive cell lines, designated 42, 47 and 56, were derived from fusions with the NSO myeloma cell line. The fourth positive cell line was derived from fusion with the NSI myeloma cell line and designated 1-28.

Other fusions were performed in the manner just described except that the fusion partner was cell line P3X63Ag8,653 (Kearney et al. (1979) J. Immunol. 123:1548-1550). ELISA screening was performed with the following layers:

1st layer: Purified Factor VIIIIC
2nd layer: Hybridoma cell supernatant
3rd layer: Horseradish peroxidase (HRP)-
labelled goat anti-mouse IgG

5 4th layer: HRP-substrate

Five positive cell lines were identified, subcloned, grown in liter scale cultures and characterized as described in Section 4. The cell lines were designated as 1B9, 2B6, 2F9, 4E5 and 5B1.

10 3. Amplification of
Positive Hybridomas

In order to be certain that each hybridoma producing anti-Factor VIII antibodies was free from cells producing other immunoglobulins and cells not producing immunoglobulins, purification by subcloning was performed. Dilutions of hybridoma cells 42, 47, 56 and 28 were seeded in microtiter plates, such that each well theoretically contained a single cell. When necessary because of low cloning efficiency, initial seeding was at two cells per well followed by a second seeding at one cell per well. After 7 to 14 days, supernatants from each colony of presumptively identical cells derived from each hybridoma cell line were screened to identify the anti-Factor VIII producing colonies. The positive subclones were then expanded to macroculture.

Multiple aliquots of each cell line were frozen in liquid nitrogen as soon as purification was complete. This provided amplification of the cultures from the original single microwell (10^2 cells in 0.2 ml) to about 10^8 cells, which corresponded to about 100 ml of medium. The cells were very sensitive during this stage of growth and improper handling could result in death of the culture. Although individual characteristics of each cell line meant that identical procedures could not be used for all four lines, the

following steps were generally employed to keep the cultures alive and growing:

1. Micro wells were approximately $\frac{1}{4}$ confluent before transfer to macroculture.
- 5 2. Pre-conditioned medium was used for the first two weeks of subculture.
3. Transfer from macro to flask culture was delayed until at least 4 ml of macroculture was grown to half-confluence.
- 10 4. Dilution of the flask culture was limited to less than 1:2 for the first few passages.

4. Characterization of Monoclonal Antibodies

To identify the immunoglobulin class of the monoclonal anti-Factor VIIIC antibodies, an immunoassay
15 for mouse IgG was carried out in which the coating antibodies were specific for immunoglobulin-subclasses. Microplates precoated with anti-isotype antibodies were obtained from Hagedorn Research Lab. The antibodies produced by hybridoma cell lines 42, 47 and 56 were
20 found to be IgG 1, while those produced by cell line 5B1 were found to be IgM.

Two classes of monoclonal antibodies were identified according to their reactivity with Factor VIIIC polypeptides using the following procedure.
25 Hybridoma culture supernatants were incubated for 12 hours at room temperature with polystyrene beads ($\frac{1}{8}$ " diameter, Precision Plastic Ball Co., Chicago) coated with affinity-purified goat anti-mouse immunoglobulin (IgG). The polystyrene beads were washed with
30 phosphate-buffered saline and reacted with purified Factor VIIIC labelled with I^{125} using the chloramine T method. The labelled proteins bound on the beads were solubilized in SDS sample buffer for gel electrophoresis and were separated on an 7.5%
35 acrylamide gel (Laemmli (1970) Nature 227:280). The gel was fixed, dried and exposed to Kodak X-Omat film with an intensifying screen for 20 hours or longer at

-80°C. The results obtained with this procedure allowed classification of the monoclonal antibodies as follows:

- Class I: react with the 80/77 kd doublet or with the 80/77 kd doublet and a 240 kd protein. These antibodies also react with a 70/67 kd doublet when thrombin-digested Factor VIII is used in the assay, indicating that the 70/67 kd doublet produced by thrombin digestion, is derived from the 80/77 kd doublet.
- Class III: react with 240, 140, 115, 92.5 kd proteins as well as with a 40 kd peptide produced by thrombin digestion of purified Factor VIIIC material.

Table 1 indicates the Class corresponding to each of the monoclonal antibodies produced by the different hybridoma cell lines.

Table 1
Classification of monoclonal antibodies

	Cell Lines
Class I:	1B9; 2B6; 2F9; 42; 47; 56; I-28
Class III:	4E5; 5B1

The antibodies were screened for Factor VIIIC coagulation inhibition activity using the conventional Bethesda assay (C. Kasper (1975) Thrombos. Diathes. Haemorrh. 34:869-782). Of the above antibodies, only those produced by cell line 56 displayed coagulation inhibition activity.

The following experiments were performed to study the association between the 92.5 kd protein and the 80/77 kd doublet, as well as to investigate the role of calcium in the association. The following ELISA assay were performed in the presence of 10 mM CaCl_2 or in the presence of the same concentration of CaCl_2 , 10 mM EGTA, and 10 mM EDTA.

- 1st layer: Monospecific anti(mouse IgG)
2nd layer: Class III monoclonal antibody (anti-92.5 kd) or Class I monoclonal

antibody (anti-80/77 kd)

3rd layer: Purified Factor VIIIC material

4th layer: HRP-human inhibitor antibody to
77/80 kd

5 The results are shown in Table 2. As Table 2
shows, a strong HRP reaction is obtained when only
calcium is present in the assay, indicating that the
4th layer (HRP-human inhibitor antibody to 77/80 kd) is
binding to the complex associated to the Class III
10 monoclonal antibody. When EGTA and EDTA are added to
the assay, HRP activity bound in the assay corresponds
only to non-specific binding. These results
demonstrate that the 92.5 kd protein is bound to the
80/77 kd doublet through calcium bridges.

15

Table 2

Role of calcium in the association
of 80/77 kd doublet to 92.5 kd protein

Antibody specificity (2nd layer)	CaCl ₂		CaCl + EGTA + EDTA	
	A ₄₉₅ sample	A ₄₉₅ <u>sample</u> A ₄₉₅ ^{NSB*}	A ₄₉₅ sample	A ₄₉₅ <u>sample</u> A ₄₉₅ ^{NSB*}
20 Non-specific binding*	0.229	1	0.068	1
Medium only**	0.248	1.08	0.67	0.99
25 Class III (5B1) (92.5 kd)	0.939	4.10	0.071	1.04
Class I (47) (80/77 kd)	1.81	7.9	0.523	7.69

* Non-specific binding (NSB) was measured by the ELISA assay
deleting the FVIIIC material.

30 ** Medium only was added instead of the monoclonal antibody (2nd
layer) in the ELISA assay.

Additional assays were performed to
characterize the monoclonal antibodies in terms of the
epitopes they recognized. Competition assays were
performed by reacting Factor VIIIC immobilized on

microtiter wells with ^{125}I -42 IgG or ^{125}I -47 IgG and unlabelled 42, 47, 56 and I-28 at various dilutions. The results showed that 47 and 42 monoclonal antibodies react with different epitopes. They also showed that it is likely that 56 monoclonal antibodies react with an epitope near the binding site of 42 monoclonal antibodies and induce a conformation change in VIIIC that enhances the affinity for 42 antibodies.

Monoclonal antibodies capable of distinguishing between various of the constituent polypeptides of Factor VIIIC are provided. Class I antibodies react with a 80/77 kd doublet or with both a 240 kd fragment and a 80/77 kd doublet. Class III monoclonal antibodies react with the 240 kd polypeptide, as well as a 92.5 kd polypeptide and its precursors. Class III antibodies show an additional reactivity with a 40 kd thrombin digestion product of the Factor VIIIC material.

Although the foregoing invention has been described in some detail for purposes of clarity of understanding, it will become obvious that certain changes and modification may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A hybridoma cell line selected from the group consisting of cell line 42, cell line 47, cell line 56, cell line 1-28, cell line 4E5, cell line 5B1, cell line 1B9, cell line 2B6, and cell line 2F9.
- 5 2. Monoclonal antibodies obtained from any of the hybridoma cell lines of claim 1.
3. Hybridoma cell lines producing monoclonal antibodies having reactivity substantially identical to any of the monoclonal antibodies of claim
10 2.
4. Monoclonal antibodies obtained from any of the hybridoma cell lines of claim 3.

